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ORIGINAL ARTICLE

# ISHAGE-based single-platform flowcytometric analysis for measurement of absolute viable T cells in fresh or cryopreserved products: CD34/CD133 selected or CD3/CD19 depleted stem cells, DLI and purified $CD56^{+}CD3^{-}$ NK cells

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**Abstract** Considering the growing use of immunotherapeutic strategies in paediatric stem cell transplantation associated with risk of graft-versus-host disease, an accurate method for the enumeration of residual T cells/kg recipient's body weight is of paramount importance. Therefore, we propose a multi colour-flow cytometric strategy for correct absolute vital T cell enumeration in manipulated cell preparations for clinical use. The gating strategy is based on the ISHAGE single-platform stem cell enumeration method in combination with experiences from lymphocyte subtyping, using low scatter, high expression of CD3 and CD45 antigens and 7-AAD staining in a no-wash-preparation with counting beads. In spiking experiments, the detection limit was determined to be at $0.7 \pm 0.5$ $CD3^{+}$ cells/μl with a minimum of 50 T cell events acquired. The cell preparations analysed contained a median absolute $CD3^{+}$ T cell number of $221 \times 10^3$ (0.09%, CD34 selected grafts, $n = 187$), $900 \times 10^3$ (0.004%, CD3/CD19 depleted grafts, $n = 15$) and $283 \times 10^3$ (0.012%, CD3 depleted/CD56 enriched NK-cells, $n = 14$), respectively. The results differed of those from conventional T cell measurement in cell products after extensive manipulation. Our method provides reliable residual T cell enumeration even at extremely low concentrations.

**Keywords** DLI · Graft engineering · Residual T cells · Single-platform method

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## 1 Introduction

T cells facilitate immune reconstitution after allogeneic stem cell transplantation and may reduce incidence and severity of infections. They also mediate both graft-versus-host (GvH) and graft-versus-leukemia (GvL) reactions [1–4]. Whereas GvL is beneficial, GvH reactions may cause severe acute or chronic disease. Therefore, in certain cases of childhood transplantation grafts are T cell depleted by methods that require considerable graft manipulation. While GvH is potentially dangerous, it is also made use of in case of rising mixed chimerism, a situation that denotes impending graft failure and relapse. Mixed chimerism can be reverted to full donor chimerism by donor lymphocyte infusion (DLI). To this purpose, lymphocytes of non-related donors may be cryopreserved and rethawed upon demand, a process that may entail extensive cell death. In haploidentical protocols, T cells and B cells are depleted for stem cell and NK cell enrichment. In all cases of pediatric cell therapies outlined, defined numbers of vital T cells are required by the clinician. Besides stem cell content, the number of vital T cells contained in a cell product may impact on its fraction actually infused or the amount of immunosuppressive therapy required. Dosages of DLI in

children are carefully calculated and only slowly escalated to minimise risk of severe GvHD. Thus, cell analysis in the context of allogeneic transplantation must reliably quantify vital T cells contained in cell preparations that have undergone extensive manipulation with resulting cell damage. In addition, T cell concentrations in these cell preparations range over three log ranks from near detection limit to above those in peripheral blood (PB).

Flow cytometric T cell measurement has been developed in the context of monitoring the progress of HIV infection [5–8]. Protocols that define lymphocytes both by light scatter and CD45 antigen expression and that incorporate fluorescent counting beads as internal standard have advanced T cell measurement considerably [7–13]. Still, these methods are intrinsically designed for fresh blood samples and disregard cell viability. Then again, stem cell enumeration according to ISHAGE guidelines includes a dead cell dye together with counting beads and cell definition through light scatter properties and antigen expression [14, 15]. We have therefore adopted this protocol to measure the concentration of T cells. Here, we present our results with this new method.

## 2 Materials and methods

### 2.1 Cell products

Samples were chosen to represent the range of cell preparations processed in our laboratory. These included clinical samples of $CD34^+$-selected ($n = 187$), $CD133^+$-selected ($n = 9$) as well as $CD3^+$ and $CD19^+$ ($n = 15$) depleted stem cell grafts; $CD3^+$-depleted, $CD56^+$ enriched NK-cell products ($n = 14$) for an immunotherapy trial; PB, both fresh ($n = 25$) and cryopreserved ($n = 30$) for DLI; and unmanipulated bone marrow grafts ($n = 35$).

Cryopreserved preparations were maintained in X-vivo 10 media (BioWhittacker, Verviers, Belgium) with 10% dimethyl sulfoxide and stored in liquid nitrogen.

Positive enrichment ($CD34^+$, $CD133^+$) of stem cells or serial $CD3^+$ and $CD19^+$ depletion as well as purification of $CD56^+CD3^-$ NK cells was done according to standard protocols using the appropriate clinical-grade CD3, CD19, CD34, CD56 and CD133 Microbeads with the automated CliniMacs system (Miltenyi Biotec, Bergisch-Gladbach, Germany) as described previously [16–20] observing good manufacturing practice.

### 2.2 Sample staining and flow cytometric analysis

A no-wash technique was processed throughout. Reverse pipetting was used where appropriate. Samples with a white blood cell count (WBC) above $30 \times 10^6$/ml were diluted with DPBS with 5% heat-inactivated FCS (Foetal Bovine Serum Gold, PAA Laboratories, Austria) to $<15 \times 10^6$/ml. The dilution factor was recorded for the calculation of the cell concentrations.

Samples were prepared in triplicates. Accurately 100 µl of thoroughly homogenised cell suspension were stained with each 20 µl of fluoresceine-isothiocyanate (FITC) conjugated anti-CD45 monoclonal antibody (mAb) (Coulter Immunotech, Marseilles, France) and of phycoerythrine (PE) conjugated anti-CD3 mAb (clone UCTH1, Coulter Immunotech). Alternatively, following $CD3^+/CD19^+$ depletion, an anti-CD3 mAb clone SK7 (BD Biosciences, Heidelberg, Germany) was used that recognises a different binding site than the T cell depletion mAb. Control samples stained with anti-CD45-FITC (clone J.33) and the appropriate isotypic PE antibodies (Coulter Immunotech) were used to exclude unspecific staining. To distinguish between dead and vital cells, 20 µl of 7-Amino-Actinomycin D (7-AAD Viability Dye, Coulter Immunotech) were added to each tube. Samples were incubated for 20 min in darkness at room temperature. Erythrocytes were lysed with 2 ml of ammonium chloride lysing solution. In case of low or no erythrocyte content, solely 500 µl of PBS were added. The samples were then stored at 4°C in darkness until measurement within one hour. Rethawed samples were analysed at once. Immediately before measurement, with the same pipette as used for the sample accurately 100 µl Flow-Count Fluorospheres (Coulter Immunotech) were added after gently shaking. All tubes were then immediately vortexed for 5 s. Either a minimum count of 50 $CD3^+$ events and at least 75.000 $CD45^+$ events were acquired or data acquisition was terminated after 7 min.

In parallel, samples were prepared for conventional T cell analysis. The cells were stained with the tetraChrome CD45/CD4/CD8/CD3 reagent (Coulter Immunotech) and measured after addition of fluorospheres as described above.

Flow cytometric analysis was performed using an EPICS XL-MCL four colour-flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with a 488 nm argon laser and 525, 575, 620, 675 nm band pass-fluorescence filters using the System II software. In addition to standardise four colour-lymphocyte analysis set up, the calibration programme for the $CD34^+$ HPC Coulter Stem Kit analysis was run weekly. The instrument settings were verified daily with Flow-Check Fluorospheres (Beckman Coulter) and twice weekly with ImmunoTrol control cells (Coulter Immunotech).

The modified ISHAGE protocol was used for the samples stained with 7-AAD, while the appropriate tetraCHROME automatic gating algorithms (Beckman

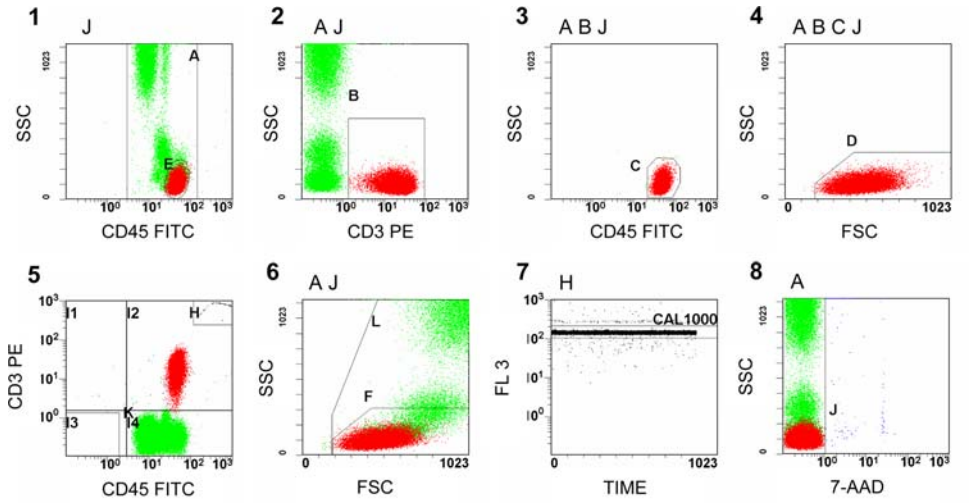


**Fig. 1** Measurement of absolute viable CD3+ T cells. From *left* to *right*, starting in the first row of plots, the gating strategy follows the ISHAGE protocol for stem cell enumeration, modified for the peculiarities of T cell measurement: Plot *1*: Region *A* is set to include all CD45+ events (leukocytes) and is gated on region *J* (plot *8*). Therefore, only 7-AAD negative (vital) events are shown in plot *1*. Region *E*: $CD45^{high}$, $SSC^{low}$ events: lymphocytes. Plot *2*: Region *B* includes all viable CD3+ leukocytes and is gated on *A* and *J*. Plot *3*: Logical AND linkage (intersection) of regions *A*, *B* and *J*. Region *C* is set to include $CD45^{high}SSC^{low}$ events (lymphocytes), only. Plot *4*: Intersection of regions *A*, *B*, *C* and *J*. Region *D* is linked with region *F* (plot *6*) so that changes in the position of region *F* will automatically be adopted by region *D*. Region *F* is used to set the low limit for FSC. The thus accepted viable CD3+ T-cells are counted in region *D*. Plot *5*: This dot plot shows all events and is used as a visual guide for antigen expression and the fluorospheres' signal. Region *K* is used to exclude CD3 and CD45 doubly negative events from acquisition. Quadrant I2 can be useful to review the lower limits of CD45 and CD3 expression, and—if necessary—to correct the position of region *A* (plot *1*) and B (plot *2*). Plot *6*: All viable CD45+ events are shown to check the lower limit of forward scatter. The amorphous region *L* is created to exclude unspecifically stained debris by low forward scatter signal. Thus, region *L* includes all viable leukocytes. The region *F* defines the lower FSC of lymphocytes and is used to position *D*. Plot *7*: Displays the fluorescent signal of the events of region *H* versus time. Region *CAL* is set to define the signal of fluorospheres and to monitor the occurrence of fluorospheres doublets, which should be less than 5% in this plot. *CAL* is the calibrator region to automatically calculate the concentrations of the events in a given gate. Steady sample flow is monitored here, too. Plot *8*: Gated on CD45+ events (Region *A*, Plot *1*), Region *J* is set to discriminate between viable and dead cells by excluding 7-AAD-positive events

Coulter) for samples with normal or low lymphocyte contents, respectively, were employed to measure conventionally stained T cells and subsets. These algorithms also use gating of $CD45^{high}$, FSC $^{low}$ and $SSC^{low}$ cells to define lymphocytes [13].

## 2.3 Spiking experiments

Precision of T cell detection was evaluated through spiking experiments with the following three samples:

(1) PB from a 17-year-old patient with relapsed non-hodgkin lymphoma three days after haploidentical SCT during aplasia without measurable T cells ($CD3^+$: not detectable and $CD45^+$:19.4/μl).
(2) Fresh PB from the donor (the patient's mother, same blood group) with 1084.5/μl $CD3^+$ cells and 4947.2/μl $CD45^+$ cells, without detectable dead cells;
(3) The remaining sample of the $CD3^+/CD19^+$ depleted apheresis product (200 μl) from the donor with a minimal T cell content ($CD3^+$: 0.7/μl; $CD45^+$: 9742.5/μl).

Informed consent of both patient and donor was obtained to use the blood and the remaining sample after completion of routine diagnostic. Based on the initial $CD3^+$ measurement, donor PB and host PB were mixed at 1:100, 1:300 and 1:1000 $CD3^+$ ratios, while donor PB and the $CD3^+/CD19^+$ depleted apheresis product were mixed at 1:10, 1:30, 1:100, 1:300 and 1:1000 and finally each sample was measured 10–12 times.

## 2.4 Comparison of samples stained with or without 7-AAD

The influence of dead cell exclusion on the $CD3^+$ relative and absolute cell counts in different cell products was tested by parallelly analysing triplicates from the same specimen with and without 7-AAD staining. Four different materials representative for therapeutical immunopreparations were used: freshly drawn PB ($n = 10$), cryopreserved donor lymphocytes ($n = 10$), positively selected $CD34^+$ stem cells ($n = 10$) and negative fractions of CD34-selections ($n = 10$). The adopted ISHAGE guidelines based protocol for T cell measurement was used throughout.

### 2.5 Calculation and statistics

Dilution factors were included in the calculations of cell concentration throughout. Cell concentrations of the samples were calculated according to

$$\text{Cells}/\,\mu\text{l} = (\text{counts}_{\text{cells}} \times \text{volume}_{\text{beads}} \times \text{concentration}_{\text{beads}})/(\text{counts}_{\text{beads}} \times \text{volume}_{\text{cells}})$$

where "$\text{concentration}_{\text{beads}}$" indicates the concentration of the fluorospheres suspension as given by the manufacturer and "volume" indicates volumes pipetted per sample.

For statistical analysis, the BIAS software was used (BIAS 7.07, epsilon verlag, Frankfurt, Germany). Graphs were created by GraphPadPrism version 4-00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Single and dual platform flowcytometric data were correlated by the Bland-Altman-Test. The mean absolute difference (bias), SD and the limits of agreement (mean absolute difference ± 2 SD) were calculated.

## 3 Results

### 3.1 Gating strategy for detection of absolute viable $CD3^+$ T cells

Based on the ISHAGE protocol for the enumeration of stem cells [14, 21] and the Beckman Coulter protocol "calculation of absolute viable $CD34^+$ HPC based on the ISHAGE Gating Strategy" the following protocol represents a strategy to measure absolute viable T cells. The data analysis strategy is based on eight dot plots with ten predefined amorphous and rectangular regions gated successively (Fig. 1). The gating strategy aims at defining T cells precisely by scatter characteristics and high expression of CD45 and CD3 antigens while excluding dead cells from analysis through 7-AAD staining. Cell concentrations are calculated by the internal standard.

### 3.2 Spiking experiments

No clotting or precipitation was seen in the mixed donor-host samples. 10 or 12 repeated samples of the patient's PB, the donor's PB, the $CD3^+/CD19^+$ depleted stem cells and of the various mixed samples yielded a precise $CD3^+$ detection in a range from 0.7 ± 0.5 to 1084.5 ± 19.8 $CD3^+/\mu l$ (Table 1). The detection limit was found to be at 0.7 $CD3^+/\mu l$.

With the limits for data acquisition set as given above, even in samples with as low a concentration as one T cell/µl, more than 50 accepted T cell events per sample could be recorded.

**Table 1** Spiking experiments for single-platform T cell enumeration, measured values

| Material | Ratio | CD3 (%)[b] | CD3 (/µl) | |
|---|---|---|---|---|
| | | | Mean ± SD | Range |
| PB donor | – | 21.92 | 1084.5 ± 19.8 | 1061–1115 |
| PB recipient | – | ND | ND | ND |
| HSC donor[a] | – | 0.007 | 0.7 ± 0.5 | ND-1.0 |
| PB donor: PB recipient | 1:100 | 16.8 | 10.2 ± 1.1 | 8.5–11.8 |
| | 1:300 | 10.4 | 3.9 ± 0.8 | 3.1–4.0 |
| | 1:1000 | 4.37 | 1 ± 0.4 | 0.7–1.4 |
| PB donor: HSC donor | 1:10 | 0.39 | 105.9 ± 4.6 | 101.8–109.0 |
| | 1:30 | 0.2 | 37.6 ± 2.5 | 35.6–39.2 |
| | 1:100 | 0.12 | 12.3 ± 1.3 | 9.8–14.3 |
| | 1:300 | 0.042 | 4.1 ± 0.8 | 3.3–4.9 |
| | 1:1000 | 0.018 | 1.7 ± 0.6 | 1.2–2.5 |

A peripheral blood sample of a healthy donor was mixed in various ratios with either a peripheral blood sample of the haploidentical recipient during aplasia post SCT without detectable T cells or the $CD3^+/CD19^+$ depleted apheresis product of the donor. Of each ratio, $n$ = 10 or 12 samples were analysed in parallel for T cell content

[a] HSC donor = CD3/CD19 depleted hematopoietic stem cells of the donor

[b] Percentage of $CD3^+$ cells of $CD45^+$ leukocytes

*ND* not detectable, below the detection limit. The value of the detection limit (0.7 $CD3^+/\mu l$) was 5–6 times above the noise

### 3.3 Evaluation of the method for residual T cell enumeration in cell products for stem cell transplantation or immunotherapy

The method was used on a total of 225 clinical samples with very low lymphocyte content (Fig 2 III, IV). In each case, at least 50 T cell events were recorded. Concentrations ranged between below the detection limit up to 8.4, 6.2 and 11.7 $CD3^+$ cells/µl for $CD3^+/CD19^+$ depleted, $CD34^+$ or $CD133^+$ selected stem cells and purified NK cells, respectively. A summary of the median percentage and the range of residual T cells as well as the absolute number of T cells in the various manipulated cell products is given in Table 2.

### 3.4 Measurement of T cells with and without 7-AAD staining

Forty samples were analysed in triplicate both with and without 7-AAD using the single-platform protocol, ten samples of each type of cell preparation as mentioned above. While freshly prepared DLI yielded a low bias for T cell detection of the two sample analysis methods (Fig. 3a), measurement of cryopreserved DLI resulted in a

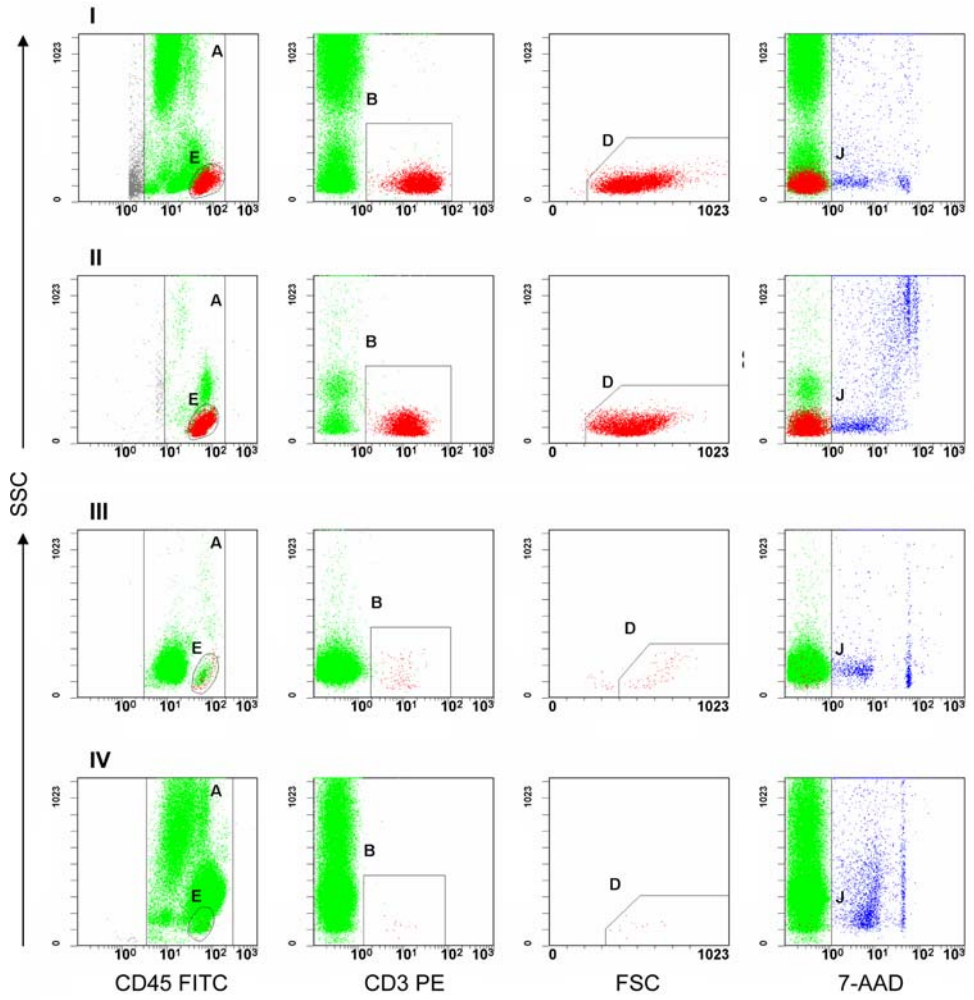


**Fig. 2** Measurement of absolute viable $CD3^+$ T cells in various cell products. Relevant plots of analysis for bone marrow (**I**), cryopreserved peripheral blood (**II**), $CD34^+$ selected peripheral blood stem cells (**III**) and CD3/CD19 depleted stem cells (**IV**) are shown

**Table 2** Percentage and absolute number of residual viable T cells in various manipulated cell products

The amount of T cells was measured by the single-platform protocol as described in materials and methods. *ND* not detectable, below the detection limit

| Material | *n* | $CD3^+$ T cells (%) of CD45+ cells | | Absolute $CD3^+$ number $\times 10^3$ cells | |
|---|---|---|---|---|---|
| | | Median | Range | Median | Range |
| CD34 selected stem cells | 184 | 0.09 | ND-0.66 | 221 | ND-1361 |
| CD133 selected stem cells | 9 | 0.15 | 0.09–0.67 | 186 | 15.4–942 |
| CD3/CD19 depleted stem cells | 15 | 0.004 | ND-0.02 | 900 | ND-4418 |
| CD3 depleted, CD56 enriched NK cells | 14 | 0.016 | ND-0.08 | 283.2 | ND-2121 |

pronounced divergence as shown in Fig. 3b. In addition, Bland-Altman analysis confirmed precise results of $CD3^+$ measurement for positive fractions of $CD34^+$-selected stem cells (bias 0.3; SD of bias 0.7). In opposition to that, negative fractions after $CD34^+$-selection yielded a higher bias of 2704, SD of bias 2507 (data not shown). The Bland Altman-analysis for $CD45^+$ leukocyte cell counts in the same samples resulted in a similar finding (data not shown).

## 4 Discussion

While many publications deal with flow cytometric T cell measurement in the setting of HIV progression diagnostics [5–8], T cell detection in stem cell grafts and immunotherapy cell preparations with their specificities seems to be disregarded.

Because the correct number of viable T cells is of high importance in allogeneic transplantation, we therefore

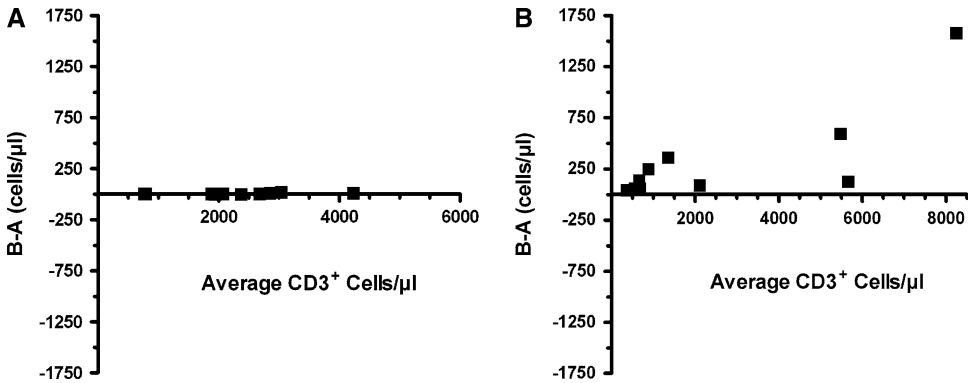


**Fig. 3** Measurement of viable $CD3^+$ T cell concentrations for DLI using single-platform measurement with and without 7-AAD in PB. Bland-Altman-Plot of fresh PB resulted in a low difference between both methods with a bias of 5.5, SD of bias 5.9 (**a**). In contrast, comparison of results from cryopreserved PB led to high differences between methods with a bias of 329, SD of bias 471 (**b**)

sought to establish a method suitable to quantify these in manipulated cell preparations that potentially contain high percentages of dead cells. The supremacy of single-platform over dual-platform measurement approaches has been discussed widely [9, 11]. In addition to eliminating an additional source of variation and error, hematologic cell counters are validated for PB, only, while the cell preparations discussed here differ widely from PB both in cell concentration, leukocyte distribution and erythrocyte content. Moreover, cell counters do not distinguish between lymphocytes and erythrocyte progenitors contained in bone marrow, producing falsely high results. Therefore, a single-platform approach using the $CD45^+$ antigen to identify leucocytes seems advisable.

Dead cell staining with 7-AAD in T cell detection has been described previously by Schmid et al. in a dual-platform approach [22]. However, they were working with PB mononuclear cells, staining with CD8 FITC and CD4 PE, while omitting the CD45 antigen. In addition Schumm et al. described a similar dual-platform protocol for enumeration of T cells in CD3/CD19 depleted grafts [23]. They define viable T cells using light scatter characteristics of lymphocytes, the absence of CD14 and CD33, negativity for PI staining and positivity for CD3 and CD4 or CD8, respectively.

For meaningful single-platform flow cytometric analysis, a lyse-no wash technique is required for erythrocyte destruction [24]. Red cell lyses can change cell characteristics, a phenomenon well known from $CD34^+$ analysis. One should be aware of the alterations in the antibody binding and scatter characteristics of the white cells in the probe [25, 26].

Small fluctuations in material with very low $CD3^+$ concentration can lead to high differences in absolute CD3 concentration. Accurate reverse pipetting technique must be observed in single-platform analysis [11, 21]. As binding capacity and, therefore, fluorescence intensity in flow cytometric analysis decreases when the same mAb is used for immunomagnetic selection and immunostaining, different clones of monoclonal antibodies must be used to obtain satisfactory fluorescence signals.

Based on the ISHAGE guidelines for stem cell enumeration we adapted a single-platform protocol for T cell measuring and tested the method with clinical samples. The spiking experiments showed satisfactory precision of T cell detection in materials with various T cell concentrations ranging from below the detection limit ($0.7 \pm 0.5$ $CD3^+/\mu l$; mean $\pm$ SD) up to $1084.5 \pm 19.8$ $CD3^+/\mu l$. In a total of 225 various cell preparations (CD34/CD133 selected or CD3/CD19 depleted grafts and highly purified NK cells), we measured T cell concentrations between "not detectable" up to 12/µl. Application of the method to samples of immunotherapeutic preparations yielded good precision and detectability of as low as 1/µl T cells with sufficient events acquired for statistic validity. As expected, a large discrepancy to conventional T cell measurement was found in samples after extensive manipulation due to their high content of dead T cells.

T cells are thought to mediate both beneficial and adverse effects in allogeneic SCT. On the one hand, they may eradicate malignant cells through GvL reactions, on the other they may lead to severe illness when attacking normal tissues. The latter GvH disease can be prevented by depleting T cells from grafts by various approaches. The limit for GvH disease induction in haploidentical SCT for pediatric and adult patients is widely accepted to be 2.5 and $5 \times 10^4$ T cells/kg recipient's body weight [27–30] respectively, regardless of the subsets of T cells. For an average graft, this limit is met at a T cell percentage and concentration near detection limit, only. In this situation, dead cells may impact significantly on the calculated number of T cells unless excluded from counting. Based on falsely determined T cell numbers, unnecessary immunosuppressive therapy may be initiated or a reduced fraction of a graft infused, endangering transplant success.

The GvH-reaction is used to the advantage of the patient when host leucocytes reappear. This situation of rising mixed chimerism may lead to gaft rejection and relapse.

Mixed chimerism has been shown to be revertible to full donor type by DLI [3]. In order not to provoke GvH disease, DLI is commenced at minimal doses, starting from as low as $25 \times 10^3$ $CD3^+$/kg body weight, then escalating if not efficient. All in all, in pediatric and adult allogeneic stem cell transplantation (matched related and unrelated SCT or haploidentical SCT) T cell amounts given range from $<5 \times 10^3$ up to $>3 \times 10^7$/kg body weight of the recipient with corresponding ranges in concentration in the cell preparations [3, 4, 27–30]. Cryopreservation of these cells for the purpose of DLI may have affected T cell viability to a great extent, depending on the materials and methods used [31–33].

In conclusion we propose a multi colour-flow cytometric strategy for enumeration of absolute viable $CD3^+$ T cells in a single platform approach. The method may help to increase safety and accuracy in transplantation and immunotherapy through correct application of the desired dose of $CD3^+$ T cells/kg body weight, especially as the matter of T cell dosage is still a subject of ongoing discussions. As further perspective, a multi-centre comparison should be performed to evaluate the method in different laboratory settings.

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